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14. ABSTRACT

Described herein is the technical information pertaining to Year 1 of a multi-year effort to determine and characterize the use of adipose-derived (fat-derived) stem cells in the treatment of epidermal (skin) wounds. Adipose tissue was successfully harvested from the nuchal fat pad of six Atlantic Bottlenose dolphins via liposuction; cells released during the digestion of the adipose tissue were analyzed for cytology, assayed for the total number of colony-forming cells, expanded in culture, differentiated into multiple cell lineages and analyzed for stem cell surface markers. Cultured cells were also cryogenically frozen for future cell therapy treatment of dolphin skin wounds.

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Annual Technical Report

Assessment of Regenerative Capacity in the Dolphin

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Prepared for the Office of Naval Research

Contract N00014-09-C-0378

For the Period 04 August 2009 to 10 October 2010

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a. **Scientific and Technical Objectives**

There is no information available on the distribution and functionality of adult stem cells in adipose tissue in the dolphin. However, it has been reported that cutaneous wounds in the dolphin heal very rapidly. We hypothesize that adult stem cells play an important role in wound healing in the dolphin. The objective of this proposal is to assess various adipose tissue depots in the dolphin for the presence of nucleated cells, to characterize those cells in order to establish their regenerative capacity and to use isolated, regenerative cells for treating wounds in a wound healing model in the dolphin. Vet-Stem will use its extensive knowledge and technical expertise to optimize the isolation of regenerative cell populations from dolphin adipose tissue, to characterize those cells with cross reactive CD markers and primers, to differentiate the cells into specific lineages and assess the therapeutic benefit of applying concentrated doses of the regenerative cells to wounds in a wound healing model. The knowledge gained from these studies will support the potential development of off-the-shelf cell-based "products" for treating a variety of pathologies and disease states in dolphins in the Navy and could extend knowledge to the treatment of sailors and other military personnel.

b. Approach

We propose to use techniques and protocols developed at Vet-Stem to characterize the nucleated cell preparations obtained from dolphin adipose tissue. The characterization will confirm the existence of adipose-derived stem cells (ASCs) in the dolphin, by demonstrating that a subset of cells from the isolated cell preparations are plastic-adherent in cell culture and these cells can be differentiated with specific media into adipogenic, chondrogenic, osteogenic and neurogenic cell lineages. This will provide the initial evidence of ASCs in dolphins. Further phenotypic characterization will be completed by assaying the cells for key surface proteins with existing immunological and molecular biological reagents for ASCs from other species. Positive identification of these existing reagents using cultured dolphin cells will prove that dolphins contain ASCs in their tissues. Cryopreserved cultured ASCs will be used as autologous cellular therapy for dolphin skin wounds. Finally, the cells will be tested for immunogenicity to develop an allogeneic (same species,universal donor cell line) model of cell therapy in the dolphin. After establishing these safe methods, we will use autologous and allogeneic ASCs to assess the therapeutic impact of applying a concentrated "dose" of regenerative cells in the wound healing model.

c. Concise Accomplishments

Ultrasound-guided liposuction was used to harvest subcutaneous adipose from the nuchal pad of six dolphins at the Navy Marine Mammal Program (NMMP). Adipose samples were enzymatically digested to release cells from the adipose tissue matrix and all six collections yielded sufficient nucleated cells to initiate primary cell cultures. A protocol was developed to optimize the number of cells released from the adipose and was found to be specific for each animal/sample. Fibroblastic colony forming unit assays were performed to enumerate the percentage of stem cells isolated. Additionally, cytological analyses were performed, and cells were cryopreserved for future autologous cell therapy use and for future cellular characterizations. Cultured cells were also plated for differentiation assays at passage number 2 and 4 and were successfully differentiated into adipogenic (fat), chondrogenic (cartilage), neurogenic (neuronal), and osteogenic (bone) cell lineages, indicating that the nucleated cells cultured from the dolphin adipose are multipotent regenerative cells. Phenotypic

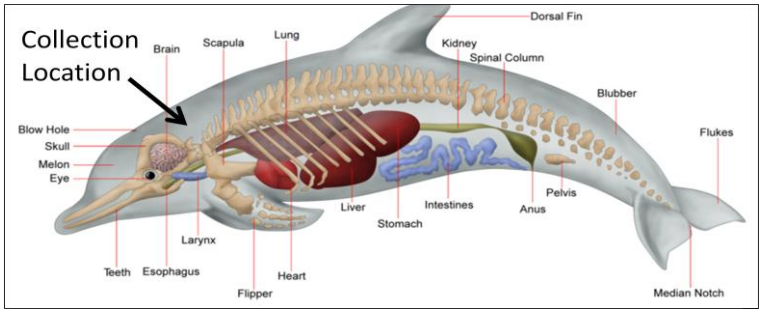
characterization of the cells using antibodies has been initiated and resulted in the positive staining of CD73 (5'-endonuclease). These results indicate that the cells isolated from the dolphin adipose are adipose-derived stem cells based upon the following: cells are plastic-adherent, differentiate into multiple cell lineages and express key surface proteins.

d. Expanded Accomplishments

Collection of Adipose from the Atlantic Bottlenose Dolphin

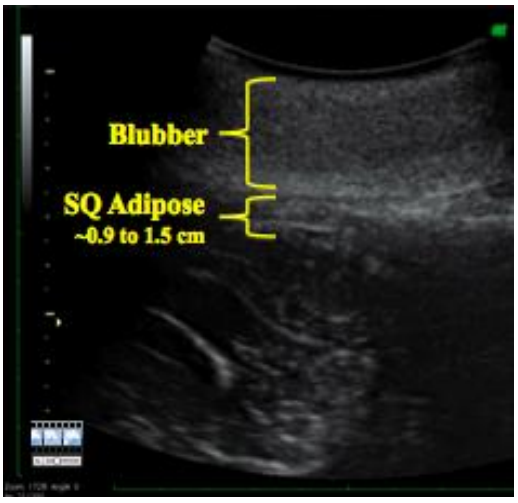
While there are multiple adipose depots within the Atlantic Bottlenose dolphin, following consultation with the NMMP veterinarians, the region identified as being the most likely to have a high density of adipose was the nuchal fat pad; an area analogous to the back of the human neck (Figure 1). The use of ultrasound was deemed the optimal method to verify the location, measure the density and aid in the placement of the liposuction cannula in the adipose depot. The difference between the various layers of muscle and adipose tissue is readily visible on the ultrasound and aids in the progression of the cannula to the adipose field (Figure 2). The actual liposuction technique is largely based on Vet-Stem's experience from equine (horse) liposuction collections.

Figure 1. Anatomical Diagram of the Atlantic Bottlenose Dolphin.



Following transport to the surgical suit, the dolphin is allowed to stabilize while respiration and heart-rate monitors are attached. The animal is given a local skin block (lidocaine) in the approximate area where the infusion cannula will be inserted. An infusion cannula with attached syringe is then used to infuse the intended adipose harvest site with tumescent solution; a solution consisting of lidocaine, epinephrine and saline. A waiting period of between 10 and 20 minutes is then required to ensure analgesia; during this time the collection area is massaged to diffuse the tumescent solution evenly throughout the area and assist in the disruption of the adipose depot connective tissue. After the designated waiting period, a harvesting cannula is attached to a 60 milliliter Toomey syringe and the cannula is then inserted into the same entry port used for the infusion. The liposuction is performed by applying a vacuum to the syringe, monitoring the progression of the cannula to the collection area (Figure 3) and continuously moving the cannula to assist in the harvest. Throughout the entire procedure, ultrasound imaging is used to ensure animal safety and proper identification of the subcutaneous adipose depot. The duration of the liposuction procedure is controlled by the

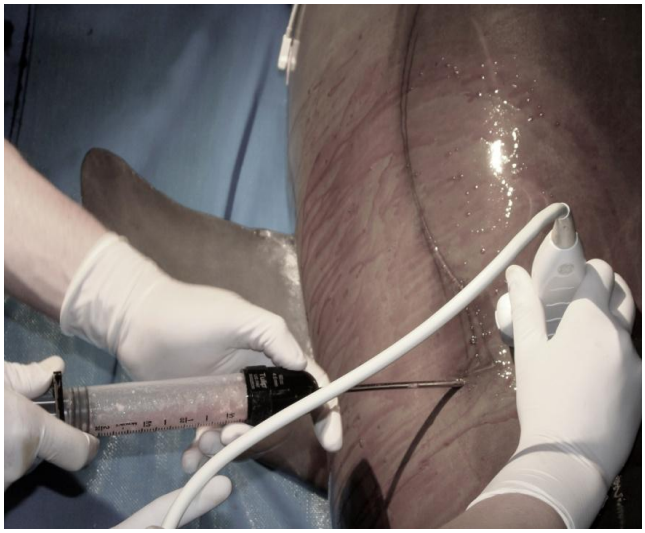
Figure 2. Ultrasound Identification of tissue layers in the dolphin.



NMMP veterinarians and is typically completed in less than 40 minutes from the time of infusion, the time at which the analgesia from the tumescent solution begins to wear off. The method described herein has resulted in the safe collection of adipose from the six dolphins; no adverse events related to this procedure have been observed or reported.

The subcutaneous adipose depth was measured during each procedure and ranged from 0.7 to 1.5 cm. As per the dolphin adipose liposuction collection schedule outlined to us by the NMMP IACUC, a total of six dolphins had adipose tissue collected via liposuction, 4 males and 2 females. The first collection performed on 02 December 2009 and the final collection was performed on 16 March 2010. Adipose sample collection information is shown later in Table 1. An additional tissue collection was performed during a dolphin necropsy on 30 April 2010. A total of six NMMP personnel, four veterinarians and two veterinary technicians, have been trained on the procedures outlined in our Quality-approved training aid, Dolphin Tumescent Liposuction Aid. NMMP veterinarians trained, supervised and assisted one another, demonstrating that this procedure could readily be deployed with field-training.

Figure 3. Photograph of Ultrasound-guided Liposuction.



Two additional adipose-containing tissues have been identified and were compared against subcutaneous adipose, which served as an experimental control. The dolphin echolocating apparatus consists of a unique composition of adipose, both on the outer edge of the dolphin lower mandible (jawbone) and in an area that is analogous to the cheek. The blubber is a specialized organ partially consisting of some adipose, which serves as an insulator to the cold water temperature and may contain a population of stem cells that will translocate based upon a sustained injury. The data from these samples is later shown Table 2.

An area of improvement for the harvesting procedure includes the use of larger diameter infusion and aspiration cannula to efficiently traverse the cannula through the various layers of epidermis, blubber and adipose. A larger cannula would allow for more rigidity in the wall of the cannula, which could lead to more consistent harvests of adipose tissue from the dolphin. A key aspect to the collection procedure is the relationship of the angle of the harvesting cannula to the adipose depot; this angle should be as shallow as possible to keep the harvesting cannula within the adipose depot for optimal harvest (see Figure 3). A more rigid cannula would allow more control of this procedure.

Using ultrasound-assisted liposuction is recommended as the optimal method to collect adipose from dolphins as the procedure is minimally invasive, has demonstrated safety and results in successful adipose harvests.

Associated tasks completed from the above section:
Milestone A003: Support Naval Marine Mammal Program in the best practices for collecting adipose tissue from a dolphin.

Milestone A010: Complete collection of dolphin adipose samples.

Digestion of Adipose Collected from Atlantic Bottlenose Dolphin

To optimize the procedure, we set out to monitor the progression of adipose tissue digestion including the use of a hemacytometer for monitoring the size of the particles in the digestate. However, particle size remained too large to properly view using a hemacytometer due to ligamenture and fibrous tissues collected during the liposuction process. Instead, qualitative observations made throughout the digestion process were used to optimize tissue digestion by removing the digestion tubes from the digestion chamber, observing the progression of digestion and stopping the digestion when appropriate. As we have seen with other species, outcomes vary greatly between animals. Therefore, there is not one set of optimal digestion parameters, per se, but rather a window of digestion time which yields both cells of high viability (greater than 65%) and high number of viable cells per gram of adipose (greater than 150,000 viable cells per gram). The data for adipose digestion is tabulated in Table 1. The average number of viable cells per gram was 357,485 with an average of percent viability of 77.7%.

Table 1. Liposuction Sample Information.

Accession Number	*Collection Date*	*Sample Weight (grams)*	*Total Viable Cell Yield*	*Viable Cells per Gram*	*Percent Viable Cells*
OBA-120209-10*	12/2/2009	6.30	546,000	86,667	86.7
OBA-012610-08	1/26/2010	12.76	3,280,000	257,053	88.2
OBA-021710-06	2/17/2010	4.62	1,546,200	338,571	75.2
OBA-022310-07	2/23/2010	5.89	4,097,700	695,705	83.1
OBA-030910-10**	3/9/2010	0.91	182,700	200,110	50.0
OBA-031610-11	3/16/2010	5.79	3,281,800	566,805	83.0
	Average	*6.05*	*2,155,733*	*357,485*	*77.7*
	Standard Dev.	*3.84*	*1,622,274*	*230,981*	*14.3*

*Sample was digested using a small adipose biopsy protocol. All other samples processed according to our standard procedure.

**Cell count was at the limit of detection; actual viability may be higher. A recount was not performed since all cells were placed directly into culture to maximize the cellular yield.

To briefly describe the digestion process, the lipoaspirate is centrifuged, the adipose is isolated, washed (Figure 4) and digested using a proprietary blend of enzymes over a time course of 20 to 25 minutes in length (Figure 5). The samples are then repeatedly washed and centrifuged to obtain a purified cell pellet (Figure 6). The total amount of adipose harvested via liposuction from each dolphin varied, but to ensure the maximum number of cells could be released, each individual sample was qualitatively-observed throughout the digestion process and digestion was halted when the majority of adipose particles were no longer visible.

Figure 4. Photographs of Harvested Lipoaspirate and Isolated Adipose Tissue.

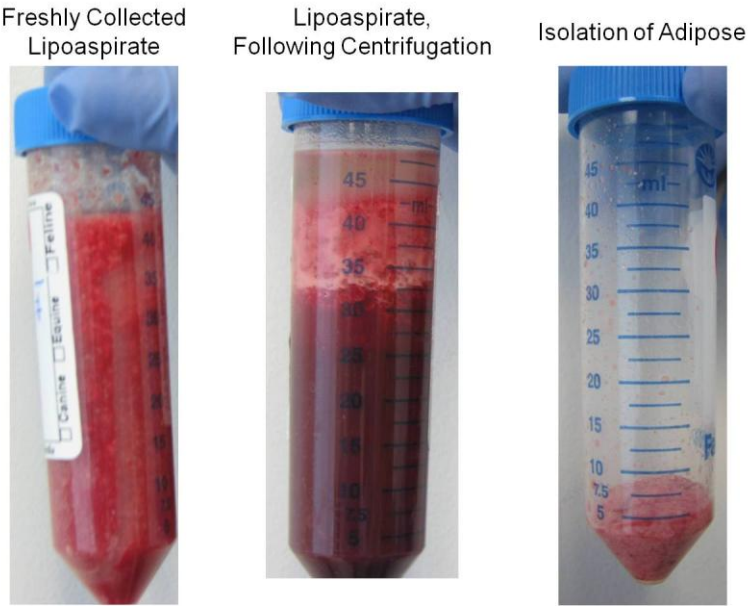
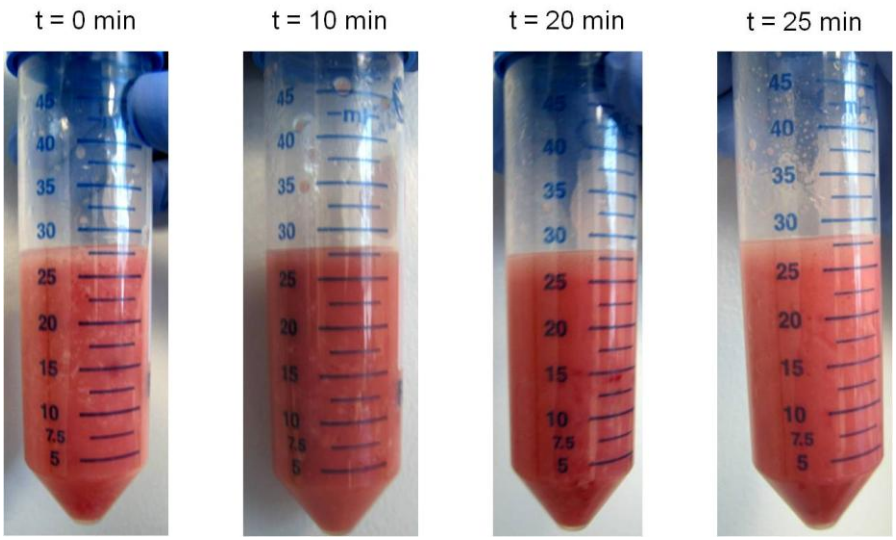
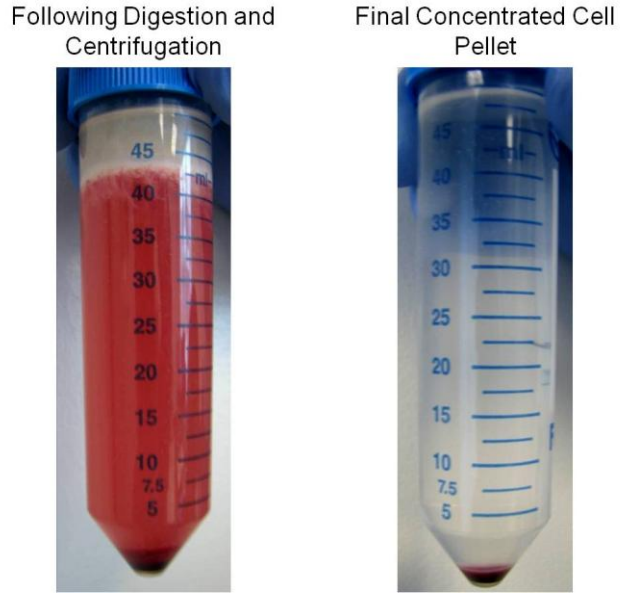


Figure 5. Photographs of the Digestion Process at Various Time Intervals.



The initial sample, OBA-120209-10, was processed differently from the remaining samples using a technique specifically developed for this contract. In brief, the adipose is centrifuged, isolated and washed, and then divided amongst multiple microcentrifuge tubes. Digestion cocktail (a proprietary blend of enzymes) was then directly added to each tube and the sample was warmed and agitated on a rocking platform. The total number of viable cells per gram for this procedure was lower than expected (86,667 viable cells per gram) and while the yield for this sample was sufficient for placing the cells directly in cell culture for expansion, all other samples were digested using our standard procedure with the previously described digestion process difference and resulted in an increased number of viable cells per gram (>200,000 viable cells per grams).

Figure 6. Completion of Adipose Digestion and Concentrated Cell Pellet.



To compare outcomes from various adipose depots, adipose samples were collected by lipectomy from three separate locations during a dolphin necropsy; mandibular adipose, subcutaneous adipose, and blubber. Tissue samples were washed, trimmed, minced and then digested, as described above, but for a total time period of 50 minutes. Following digestion, the blubber contained a large amount of ligamenture which could not be digested, whereas the mandibular adipose and the subcutaneous adipose were readily digested. In this single set of comparisons, blubber contained the least number of cells per gram and subcutaneous adipose contained the highest number of cells per gram (Table 2). The total viable cells per gram for these tissues were very low (<50,000 cells per gram) and is most likely due to the time of harvest from the dolphin necropsy. However, adherent cells were isolated from the tissues, indicating these areas may be useful for future samples. Additionally, this data further supports the use of subcutaneous adipose for isolating adipose-derived stem cells.

Table 2. Results from Dolphin Necrospy Adipose Collection Performed on 30 April 2010.

Accession Number	*Collection Location*	*Sample Weight (grams)*	*Total Viable Cell Yield*	*Viable Cells per Gram*	*Percent Viable Cells*
OBA-043010-01	Blubber	48.66	182,000	3,740	82.5
OBA-043010-02	Subcutaneous Adipose	31.92	1,484,800	46,516	87.1
OBA-043010-03	Mandibular Adipose	37.40	1,282,600	34,294	91.7

With the exception of the initial sample, all dolphin liposuction collections have been processed according to Vet-Stem's liposuction protocol, with the addition of monitoring the digestion stop. This protocol has been used to successfully release cells from over 200 equine liposuction samples. Due to the large variability between animals, the recommended protocol for dolphin adipose tissue samples collected via liposuction is to use our commercial protocol up to the digestion process and then to directly monitor the adipose digestion to ensure completion of the digestion process, maximizing the number of cells released from the adipose.

Associated tasks completed from the above section:

Milestone A004: Initiate dolphin adipose tissue digestion.

Milestone A009: Initiate optimization of digestion process, if appropriate.

Milestone A011: Establish the digestion profile for obtaining viable cells from dolphin tissue.

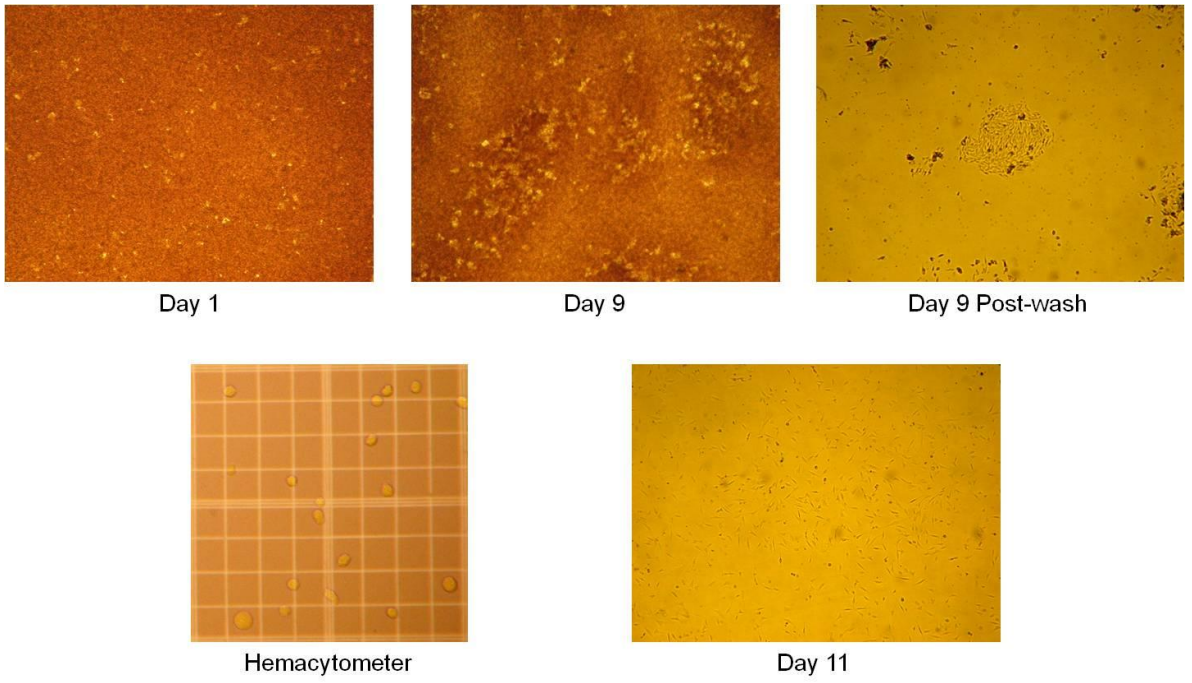
Characterization of Cells Released from the Digestion of Dolphin Adipose

To characterize cells released from dolphin adipose during the digestion process, an hierarchical schema was employed to ensure that, at a minimum, dolphin cells could be expanded in culture to obtain enough cells to treat the dolphins autologously while characterizing the cells as best possible. Although not accepted universally, the International Society for Cellular Therapy (ISCT) has established minimal criteria for defining multipotent mesenchymal stem cells (Dominici M, et al., 2006):

1. Cells must adhere to plastic when placed in culture conditions;
2. Cells must express the Cluster of Differentiation (CD) markers CD73, CD90 and CD105 and lack expression of CD34, CD45, CD14, CD11b, CD79, CD19 and HLA-DR (major histocompatibility class II);
3. Cells must differentiate into adipocytes, chondroblasts and osteoblasts in vitro.

To begin demonstration that the cells isolated from the digestion of dolphin adipose are stem cells, nucleated cells were expanded in culture. Cells were seeded directly onto cell culture flasks at an initial seeding density of 13,333 cells per square centimeter. Since red blood cells are collected during centrifugation with the mesenchymal stem cells, the initial culture seeding contains a large number of red blood cells. Typical growth and passage observation of the stem cells is shown in Figure 7, which encompasses the typical culture at Day1 through the first passage at approximately Day 10. The cultured cells measured from 11 to 29 microns, as calculated using a hemacytometer. Throughout the culture process, the cells remained the characteristic spindle-shape of cultured stem cells and remained as such throughout all 6 passages. Cells were expanded in mesenchymal stem cell media, subcultured when cells reached approximately 80-90% confluence and plated into new flasks at a minimum seeding density of 4,000 cells per square centimeter. The average doubling time for all dolphin cultures was found to be 1.14 +/- 0.31 days with individual culture doubling times ranging from 0.78 to 2.09 days (Table 3). All cultured cells which are to be used for autologous treatment have undergone rigorous sterility testing according to the United States Pharmacopeia <71> and Mycoplasma testing to ensure patient safety.

Figure 7. Cell Culture Expansion of Dolphin Stem Cells.



A “direct-to-culture” procedure was also performed, whereby a small volume of the undigested lipoaspirate was placed directly into culture media; cells were expected to slough off the adipose particles and grow directly on the culture flask. However, over the course of 2 weeks, no cells were observed to have adhered to the flask and the experiment was terminated.

When a cellular yield of sufficient quantity was obtained, cells released from the digestion of the harvested adipose tissue were submitted for cytological analysis. All cytology counts were performed on a Beckman Coulter AcT Diff hematology analyzer, which can discretely analyze the red blood cells and multiple white blood cell types. In all freshly-digested dolphin samples submitted for analysis, the percent of segmented neutrophils and lymphocytes is much higher than would be expected (Table 4); it is suspected that the software analysis package has incorrectly categorized the cells and we are in the process of coordinating with the NMMP to obtain blood cell counts taken prior to or during the adipose harvest for comparison.

Table 3. Doubling Time of Dolphin Stem Cell Cultures.

Accession Number	Passage Number	Doubling Time (Days)	Average	Std. Dev.
OCP-120209-11	2	1.38	1.54	0.39
	3	2.09		
	4	1.16		
	5	1.79		
	6	1.27		
OCP-012610-09	2	0.98	0.96	0.18
	3	0.71		
	4	0.87		
	5	1.16		
	6	1.08		
OCP-021710-09	2	1.06	0.96	0.11
	3	0.78		
	4	1.04		
	5	0.95		
	6	0.93		
OCP-022310-09	2	0.87	1.03	0.17
	3	0.85		
	4	1.00		
	5	1.20		
	6	1.20		
OCP-030910-11	2	0.83	1.19	0.29
	3	1.14		
	4	1.05		
	5	1.33		
	6	1.61		
OCP-031610-12	2	1.27	1.18	0.28
	3	1.16		
	4	1.06		
	5	1.58		
	6	0.82		
Cultures Combined	***2***	***-***	***1.07***	***0.22***
	3	***-***	***1.12***	***0.51***
	4	***-***	***1.03***	***0.09***
	5	***-***	***1.34***	***0.30***
	6	***-***	***1.15***	***0.28***
	All Passages	***-***	***1.14***	***0.31***

Table 4. Cytological Results from Dolphin Adipose Tissue Digestion.

Accession Number	White Blood Cells (K/uL)	Red Blood Cells (M/uL)	Mono-nuclear Percent	Segmented Neutrophil Percent	Lymphocyte Percent	Monocytes Percent	Eosinophils Percent	Number of Platelets (K/uL)
OBA-012610-08	2.5	0.17	4	73	23	1	3	5
OBA-021710-06	3.3	0.08	8	64	28	0	0	13
OBA-022310-07	2.1	0.18	2	69	19	1	9	7
OBA-031610-11	0.6	0.23	7	62	24	3	4	2
Average	***2.13***	***0.17***	***5.3***	***67.0***	***23.5***	***1.3***	***4.0***	***6.8***
Standard Dev.	***1.13***	***0.06***	***2.8***	***5.0***	***3.7***	***1.3***	***3.7***	***4.6***

To better quantify the actual number of stem cells present in the dolphin adipose, processes which yielded cells of sufficient quantity were plated for CFU-f analysis. Cells are seeded in culture wells using serial dilutions and expanded for a period of 7 to 10 days to determine the percentage of cells which can replicate and form colonies. The average percent CFU-f was found to be 0.13%, about 1 adherent cell in 769 cells (Table 5). CFU-f analyses were also successfully initiated for 2 of the 3 adipose depots from the dolphin necropsy (subcutaneous and mandibular adipose) and are included in Table 5. These numbers compare with literature on human adipose CFU-f assays.

Table 5. Colony-Forming Unit-Fibroblastic (CFU-F) Assay Data.

Accession Number	***Collection Location***	***Percent CFU-f***	
		Average	***Standard Deviation***
OBA-012610-08	Nuchal Pad Liposuction	0.17%	0.07%
OBA-021710-06	Nuchal Pad Liposuction	0.19%	0.16%
OBA-022310-07	Nuchal Pad Liposuction	0.11%	0.06%
OBA-031610-11	Nuchal Pad Liposuction	0.03%	0.01%
OBA-043010-02	Necropsy - Subcutaneous Adipose	1.04%	0.24%
OBA-043010-03	Necropsy - Mandibular Adipose	0.33%	0.28%
Live Dolphin Samples Combined (n=4)		**0.13%**	**0.11%**

Enough cells were present from 4 of the 6 adipose samples to perform cryopreservation of freshly isolated nucleated cells using a proprietary mixture of serum and dimethyl sulfoxide. Cryopreservation of cultured cells from all 6 dolphins has been successfully accomplished. Thus far, cryopreserved doses have been recovered from two separate dolphins; the percent recovery was 93.5% with 95.8% viable cells and 82.5% with 96.5% recovery, respectively for the two recovered doses.

Dolphin cultured cells expanded to passage 2 and 4 have been successfully differentiated into multiple cell lineages. The cultured cells respond to the respective induction media and have differentiated into adipogenic (fat), chondrogenic (cartilage), osteogenic (bone) and neurogenic (nerve) cells. Photographs of these cells are shown below (Figure 8 through Figure 12). In brief, cells are seeded in culture plates and expanded to the required confluence. Specific induction media is then added to the wells, with fresh media added every 3-4 days. When the required time course is complete, cells are fixed and stained for protein residues and/or cell morphology. The differentiation of cells at passage 6 is currently in progress; however, our progress thus far indicates that the ability of the cell to differentiate is not limited by the passage number. The ability of these cells to differentiate is further indication that these cells are mesenchymal stem cells.

Figure 8. Adipogenic Differentiation of Passage 2 (P2) and Passage 4 (P4) Cultured Dolphin Cells.

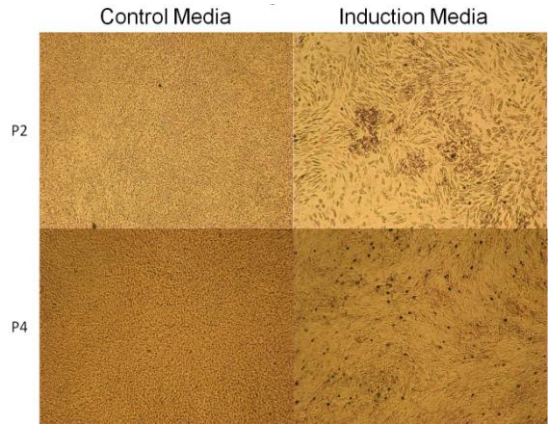


Figure 9. Chondrogenic Differentiation of Passage 2 (P2) and Passage 4 (P4) Cultured Dolphin Cells.

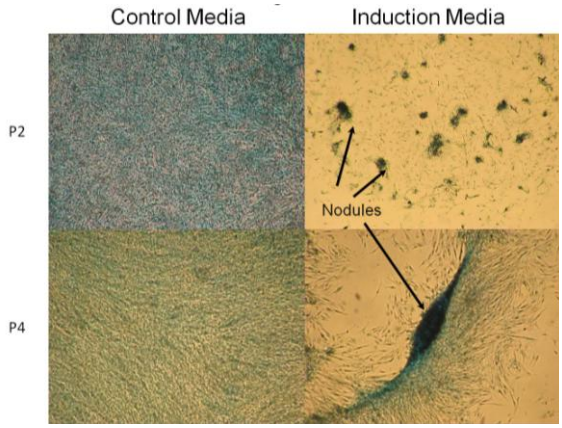
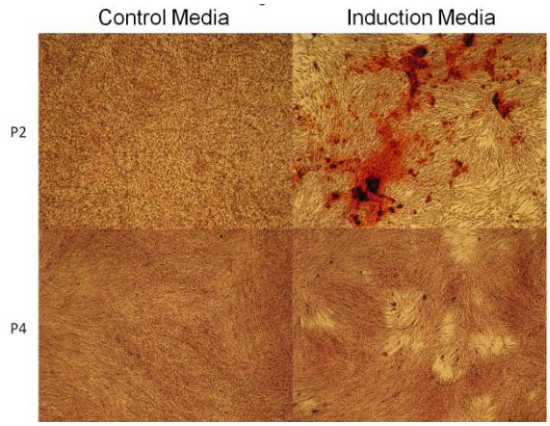


Figure 10. Osteogenic Differentiation of Passage 2 (P2) and Passage 4 (P4) Cultured Dolphin Cells.



Phenotypic characterization of the cells remains the final criteria to prove that cells isolated from dolphin adipose are mesenchymal stem cells. These phenotypic markers, termed CD markers for Cluster of Differentiation, are based upon the identification of key surface proteins expressed on the surface of the subject cells. There is little to no information available on antibodies which may be useful in successfully characterizing adipose-derived stem cells from the dolphin. Therefore, protein homology was analyzed using the BLAST algorithm (Basic Local Alignment Search Tool; www.ncbi.nlm.nih.gov/BLAST) against known sequences. Additionally, when appropriate, taxonomy was taken into consideration, as the protein sequences in the same phylum should have closer protein homology.

Figure 11. Neurogenic Differentiation of Passage 2 (P2) Cultured Dolphin Cells.

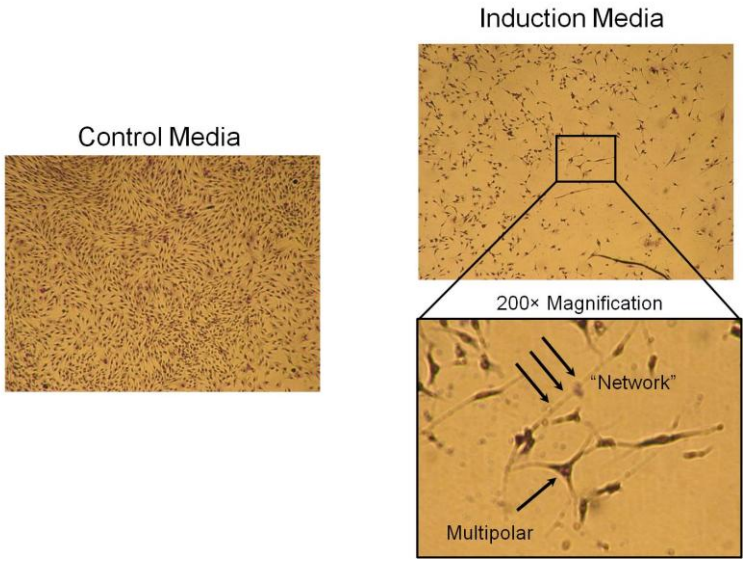
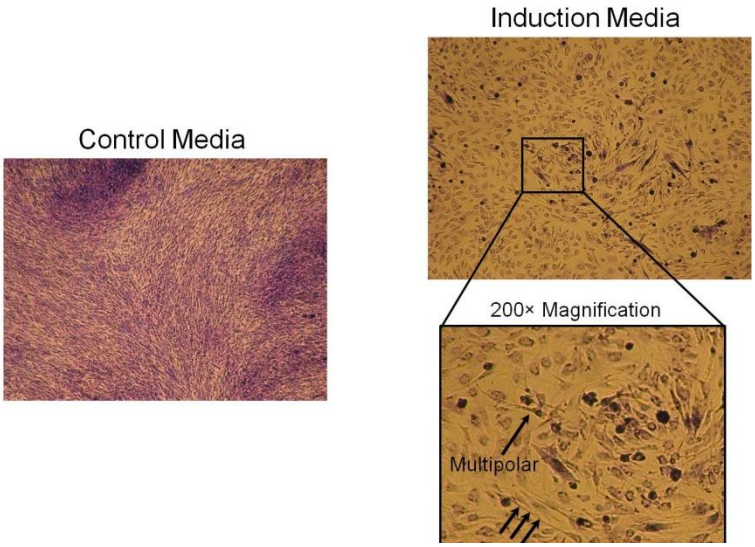


Figure 12. Neurogenic Differentiation of Passage 4 (P4) Cultured Dolphin Cells.



Using the ISCT recommendations for mesenchymal stem cells, the following human antibodies have been tested via flow cytometry on cultured dolphin cells from passage 4: CD73 (5'endo-nuclease), CD90 (thymocyte differentiation antigen 1) and CD105 (endoglin). The ISCT guidelines also recommend that cells lack certain phenotypic markers; however, determining the antibodies that will stain negative against cells that may or may not express the epitope will be difficult to prove until we have shown that at least one dolphin tissue (i.e. blood) will react positively. Cultured cells from a single dolphin were recovered and incubated with the selected antibodies or isotype controls as indicated in the figures below. A LIVE/DEAD dye was added

to all cells to enumerate the total percent of live cells; which was above 98% in all instances (Figure 13 – FSC v SSC). The intensity plots for the fluorescently-labeled antibodies are shown in Figure 14, with the isotype controls shown in Figure 15. By comparing the intensity plots of the same fluorophore, there is a large population shift in the CD73-labeled antibody (left panel in Figure 14).

Figure 13. Typical scatter plot of forward scatter (FSC-A) versus side scatter (SSC-A) for flow cytometer gating (left panel). Intensity plot of the number of live cells (right panel).

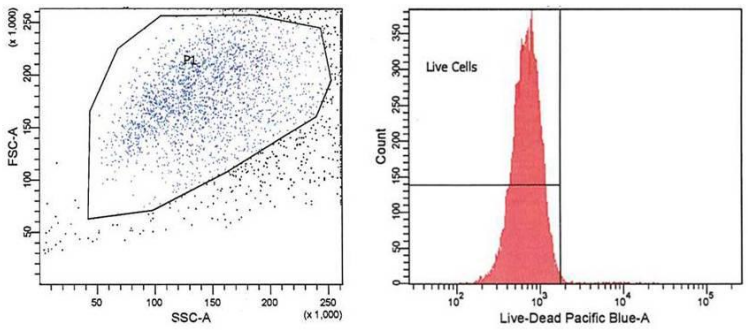


Figure 14. Intensity plots for the CD Marker Antibodies: CD73 (left panel), CD90 (middle panel) and CD105 (right panel).

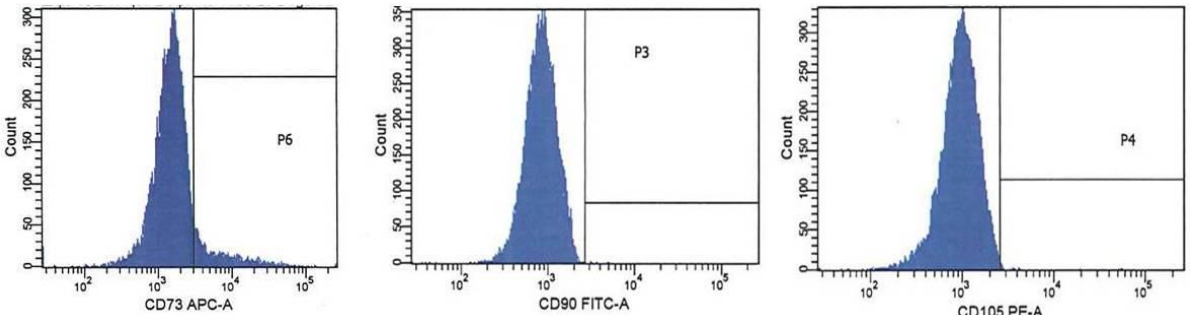
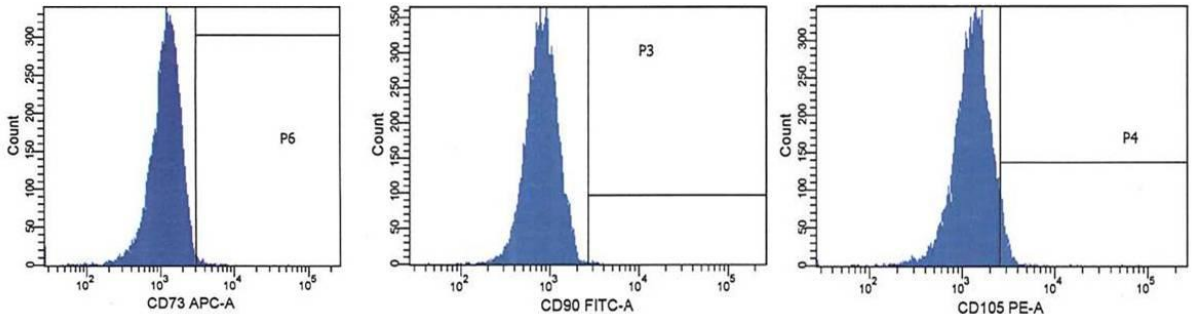


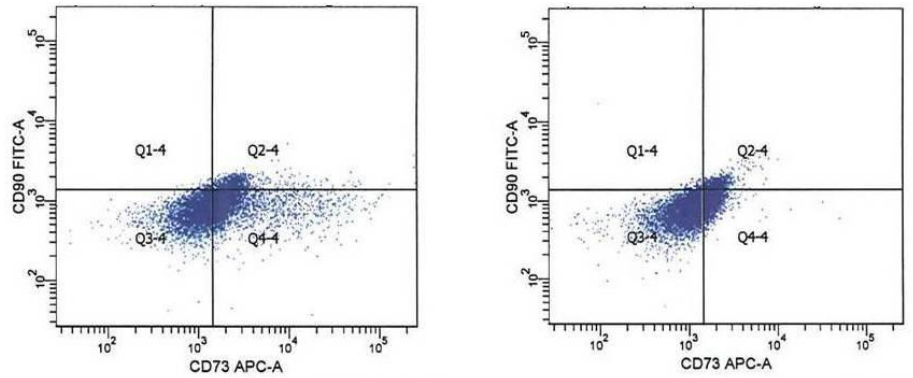
Figure 15. Intensity plots for antibody isotype controls of CD73 (left panel), CD90 (middle panel) and CD105 (right panel).



Scatter plots were then generated for CD90 vs. CD73 (Figure 16) and CD105 vs. CD73 (Figure 17) to visualize dual-staining. Cells which are negative for both antibodies will remain in the lower left quadrant (Q3), whereas cells which stain for both antibodies will be plotted in the

upper-right quadrant (Q2). Cells which stain for a single antibody will be plotted in either the upper-left (Q1) or lower-right quadrant (Q4) depending on the corresponding axis. In both Figure 16 and Figure 17, a subset of cells stains for CD73, as compared to the isotype control, and appears to account for approximately 11% of the cellular population (difference in Q4 intensities in Figure 17). Based on these analyses, it appears that CD73 is the only antibody to bind; whereas the intensities of CD90 and CD105 do not exhibit cellular binding and antibodies directed towards different epitopes will be used in upcoming characterizations.

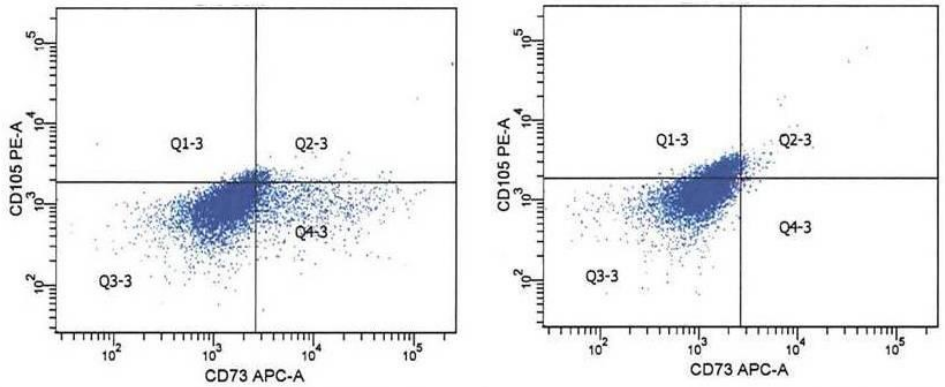
Figure 16 Scatter plot comparisons of CD90 vs. CD73 antibodies (left panel) and isotype controls (right panel).



Population	#Events	%Parent	%Grand...	CD90 Fl... Mean	CD73 A... Mean
Q1-4	48	0.5	0.5	1,501	1,204
Q2-4	953	10.3	10.2	1,625	4,690
Q3-4	3,975	42.8	42.4	709	993
Q4-4	4,315	46.4	46.0	897	3,978

Population	#Events	%Parent	%Grand...	CD90 Fl... Mean	CD73 A... Mean
Q1-4	79	0.9	0.8	1,502	1,212
Q2-4	714	7.7	7.6	1,657	2,269
Q3-4	5,852	63.1	62.5	722	960
Q4-4	2,629	28.3	28.1	962	1,845

Figure 17 Scatter plot comparisons of CD105 vs. CD73 antibodies (left panel) and isotype controls (right panel).



Population	#Events	%Parent	%Gran...	CD105... Mean	CD73 ... Mean
Q1-3	256	2.8	2.7	2,053	2,161
Q2-3	262	2.8	2.8	2,660	8,106
Q3-3	7,681	82.7	81.8	964	1,413
Q4-3	1,092	11.8	11.6	1,062	11,092

Population	#Events	%Parent	%Gran...	CD105... Mean	CD73 ... Mean
Q1-3	1,726	18.6	18.4	2,276	1,747
Q2-3	148	1.6	1.6	4,139	4,180
Q3-3	7,356	79.3	78.6	1,170	1,144
Q4-3	44	0.5	0.5	1,525	3,075

Associated tasks completed from the above section:

Milestone A005: Initiate cryopreservation of released cells from digestion of dolphin adipose tissue.
Milestone A006: Initiate cytological analysis of released cells from digestion of dolphin adipose tissue.
Milestone A007: Initiate culturing of cells obtained from digestion of dolphin adipose tissue.
Milestone A008: Initiate CFU-F assessment of cells obtained from digestion of dolphin adipose.
Milestone A012: Initiate differentiation of cultured dolphin ASCs (adipose-derived stem cells) into neuro-, osteo-, chondro-, and adipogenic cell lineages.
Milestone A013: Establish the differentiation profile of dolphin ASCs with respect to neuro-, osteo-, chondro- and adipogenic cell lineages.
Milestone A014: Initiate CD marker analysis of cultured dolphin ASCs with existing reagents.
Milestone A016: Initiate culturing of cells obtained during Year 1 Objective 3 to obtain cells at passage 4 and passage 6.
Milestone A021: Perform differentiation assessment of samples from passage 4 and passage 6.

References:
Dominici M; Le Blanc K; Mueller I; Slaper-Cortenbach I; Marini Fc; Krause Ds; Deans Rj; Keating A; Prockop Dj; Horwitz Em. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular therapy position statement. *Cytotherapy*, (2006) Vol. 8, No. 4, pp. 315-7.

e. Work Plan

We are currently in the process of completing the differentiations of passage number 6 cultured cells for adipogenic, chondrogenic, osteogenic and neurogenic cell lineages. Vet-Stem will continue to identify antibodies which stand the best chance of binding to dolphin epitopes by performing protein-BLAST analysis with known species that are more closely-related to the dolphin than the human and the mouse. In addition, other dolphin tissues such as blood will be tested to determine the degree of cross-reactivity of the identified antibodies. The results from these analyses will also aid in the selection of a microarray system to be used for primer generation and fluorescence in situ hybridization (FISH) analysis of the cultured cells and from tissue biopsies.

To genetically characterize the cells, Vet-Stem is currently in the process of evaluating cDNA microarrays for the determination of key pathways involved in cell differentiation and wound healing. Multiple stem cell microarrays are currently available, which contain known wound healing, adipogenic, chondrogenic, osteogenic and neurogenic genes. These arrays could prove useful if the dolphin sequence is considerably similar to the published human and mouse genomes. The Baylor School of Medicine has sequenced the dolphin genome and it has been published as a contiguous sequence and further BLAST analysis of human genes versus dolphin sequences will be analyzed to determine which gene microarray to employ. Sequences from the gene array will be used for primer generation in quantitative PCR for positive identification of gene expression. These primers will also be used to determine the cell fate of the stem cells in the wound healing model.

We are awaiting our latest IACUC proposal submission begin the next phase of the studies; wound healing in live dolphins. Cryopreserved doses have passed our internal quality control testing for sterility and are available for use in the donor dolphin. Next, the immunogenicity of the cultured dolphin stem cells will be tested using a mixed lymphocyte reaction assay. The results from these assays will determine how to proceed using an allogeneic (same species,

different donor) model of cell therapy. If deemed safe, stem cells from one dolphin maybe used to aid in the treatment of another dolphin.

f. Major Problems/Issues

There have been no major problems encountered thus far, but the extent of antibody cross-reactivity will limit the degree of analysis of CD markers in the cultured stem cells.

g. Technology Transfer

This ONR-funded project has provided technology to Vet-Stem in some of the following internally-funded programs:

Clinical trials in allergic skin disease.

Further characterization of stem cell markers useful for equine and canine applications.

Vet-Stem is proceeding with the development of an advanced adipose collection device, partially as a result of the liposuction procedural difficulties encountered with dolphins. This device is currently being tested in equine and canine samples.

Additional commercial cases from animals with skin disease or diseases with related mechanisms of progression have benefited. These include renal, cardiac, inflammatory bowel syndrome and liver disease.

Vet-Stem has established pilot cooperative research and development agreements with Dr. John Morley of St. Louis University. This cooperative research agreement was a result of the 2010 Office of Naval Research Biosciences Program Review. An initial study of rodent adipose has been completed; the next phase will involve the use of cell therapy treatment to rodents with Alzheimer's disease.

There are no plans for Technology Transfer of ONR-funded research and development in the upcoming year.

h. Foreign Collaboration and Supported Foreign Nationals.

None.

i. Productivity

Invited Speaker – 2010 Navy Marine Mammal Program Summit, San Diego, CA, March 23, 2010. "Regenerative Cells: Use of Adult Stem Cells in Marine Mammals."

Invited Speaker – 2010 Naval Biosciences Program Review, Alexandria, VA, June 9, 2010. "Assessment of Regenerative Capacity in the Dolphin."

Plans are in development for the presentation of the adipose collection and processing techniques at selected marine mammal scientific symposia. Once completed, the cell

characterization methods and outcomes will also be slated for presentation to share the findings with other marine mammal research groups. At least two publications are planned over the coming two years in peer-reviewed journals to present the findings of this ONR funded research.